

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 August 2007 (30.08.2007)

PCT

(10) International Publication Number
WO 2007/098201 A2

(51) International Patent Classification:
C07K 14/245 (2006.01)

(21) International Application Number:
PCT/US2007/004513

(22) International Filing Date:
16 February 2007 (16.02.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/773,658 16 February 2006 (16.02.2006) US

(71) Applicant (for all designated States except US): **THE HENRY M. JACKSON FOUNDATION** [US/US]; 1401 Rockville Pike, Suite 600, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SMITH, Michael, J.** [US/US]; 14134 Castle Blvd., Apt. 101, Silver Spring, MD 20904 (US). **O'BRIEN, Alison, D.** [US/US]; 5514 Charlote Rd., Bethesda, MD 20817 (US). **TEEL, Louise, D.** [US/US]; 915 McCeney Ave., Silver Spring, MD 20901 (US). **MELTON-CELSA, Angela, R.** [US/US]; 20516 Brandywine Court, Sterling, VA 20165 (US).

(74) Agents: **SMYTH, Robert** et al.; Morgan Lewis & Bockius LLP, 1111 Pennsylvania Avenue, NW, Washington, DC 20004 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/098201 A2

(54) Title: SHIGA TOXOID CHIMERIC PROTEINS

(57) Abstract: A chimeric Shiga toxoid according to the invention contains an enzymatically- inactivated StxA subunit and a native StxB subunit. This hybrid Shiga toxoid induces the production of broadly cross-reactive species of antibodies against Shiga toxin following immunization. The StxA subunit is modified so that it is enzymatically inactive. The invention thus encompasses the Shiga toxoid or fragments thereof and the nucleic acid sequence of the Shiga toxoid or fragments thereof. The invention further encompasses the production of a Shiga toxoid, the production of antibodies using the Shiga toxoid and methods of productions, and an immunogenic composition containing the Shiga toxoid.

044508-5015-WO
2694582.2

Shiga Toxoid Chimeric Proteins

Field of the Invention

The present invention relates to a chimeric Shiga toxoid, which can be used to vaccinate against Shiga toxins.

Acknowledgment of Federal Support

This present invention arose in part from research funded by federal grant NIH AI20148-23.

Related Application

This application claims the benefit of U.S. Provisional Application 60/773,658, filed February 16, 2006, which is incorporated by reference in its entirety.

Background of the Invention

In the United States, Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are the most common cause of infectious bloody diarrhea (Rangel *et al.* (2005) *Emerg. Infect. Dis.* 11, 603-9) and account for about 110,000 infections per year (Mead *et al.* (1999) *Emerg. Infect. Dis.* 5, 607-25). The majority of Stx-mediated disease is attributable to a subset of STEC, the enterohemorrhagic *E. coli*, which include the prototypic serotype O157:H7. The hemolytic uremic syndrome (HUS) is a serious sequela of STEC (particularly O157:H7) infection that is characterized by hemolytic anemia, thrombocytopenia and renal failure, especially amongst the most vulnerable patients - children and the elderly. The fatality rate in those who experience HUS is five to ten percent, with the potential for residual kidney and neurological damage among survivors. Therapy for STEC-infections includes supportive care, rehydration and kidney dialysis (Andreoli *et al.* (2002) *Pediatr. Nephrol.* 17, 293-8; Klein *et al.* (2002) *J. Pediatr.* 141, 172-7; and Tarr *et al.* (2005) *Lancet* 365(9464), 1073-86). No interventional therapy or vaccine is currently available. Furthermore, antibiotic treatment is contraindicated due to the increased risk of HUS (Wong *et al.* (2000) *N. Engl. J. Med.* 342, 1930-6) that may result from induction of the lytic cycle of the toxin-converting phages that encode Stxs in *E. coli*.

There are two main types of Stxs. Members of the first type, Stx produced by *S. dysenteriae* type 1 and Stx1 produced by *E. coli*, are virtually identical. The second type, Stx2 is also encoded by *E. coli*; however, it is not cross-neutralized by polyclonal antisera against Stx1, or vice versa (O'Brien *et al.* (1984) *Science* 226(4675), 694-6). Variants of each Stx serogroup exist (e.g., Stx1c, Stx1d, Stx2c, Stx2d, Stx2d-activatable, Stx2e, Stx2f) (Melton-Celsa *et al.*

044508-5015-WO
2694582.2

(2005) EcoSal-Escherichia coli and Salmonella: Cellular and Molecular Biology, ASM Press, Chapter 8.7.8) but they remain neutralizable by polyclonal sera to the prototype toxin (Schmitt *et al.* (1991) Infect Immun 59, 1065-73; Lindgren *et al.* (1994) Infect. Immun. 62, 623-31). Stxs are complex holotoxins with an AB₅ composition. They have an enzymatically active (A) subunit and a binding domain (B) composed of five identical B proteins of about 7.7 kDa each that form a pentamer. The A subunit is a ~32 kDa protein that is asymmetrically cleaved by trypsin or furin into the A1 subunit (about 27 kDa) and the A2 peptide (about 5 kDa) that remain associated through a disulfide bond. The mature A and B subunits of Stx1 and Stx2 have 55 % and 61 % identity and 68 % and 73 % similarity, respectively. Despite the amino acid sequence differences, the crystal structures of the holotoxins are remarkably similar (Fraser *et al.* (1994) Nat. Struct. Biol. 1, 59-64; Fraser *et al.* (2004) J. Biol. Chem. 279, 27511-7) and the toxins have the same mode of action. The A1 subunit contains the enzymatically active region, an N-glycosidase that removes an adenosine residue from the 28S rRNA from the 60S ribosome. This alteration halts protein synthesis and kills the intoxicated cell. The A2 peptide traverses the B pentamer to tether the holotoxin together non-covalently. The B pentamer binds the eukaryotic receptor globotriaosyl ceramide (Gb3) or Gb4, as is the case for Stx2e.

Efforts to develop vaccines protective against both Stx types have thus far been frustrating. Stxs are extremely potent and inactivation of the enzymatic activity is necessary to utilize the holotoxins as vaccines. One alternative is to use the B subunits to elicit antibodies that block binding of the B pentamer to the GB3 cellular receptor. This approach has been successful with StxB1 to raise protection against Stx1 challenge, but immunization with the StxB2 subunit is ineffective in protecting against Stx2. Furthermore, passive immunization of mice with anti-StxA2 monoclonal antibody protects mice from the effects of infection with Stx2-producing strains while anti-StxB1 monoclonal antibody is not protective against such a challenge (Wadolkowski *et al.* (1990) Infect. Immun. 58, 2438-45; Lindgren *et al.* (1993) Infect. Immun. 61, 3832-42). However, mice injected with an otherwise lethal dose of Stx1 or Stx2 are protected by passive immunization with anti-StxB1 or anti-StxA2, respectively. The toxicity of the StxA subunits is greatly abrogated without the B pentamer binding domain and there is evidence that vaccines composed of StxA1 and StxA2 offer homologous toxin protection in rabbits (Bielaszewska *et al.* (1997) Infect. Immun. 65, 2509-16). However, for safety, inactivation of enzymatic activity would be necessary for use of an A subunit vaccine in humans. Subunit vaccines in general are less desirable from the perspective that holotoxin is likely to provide a broader spectrum of protective antibodies than a subunit vaccine.

Protection against toxin-mediated diseases by immunization with toxoid (inactivated holotoxin) vaccines is successful for tetanus and diphtheria. Unfortunately, chemical inactivation

044508-5015-WO
2694582.2

of Stxs with formaldehyde or glutaraldehyde is an ill-defined chemical process that can result in residual toxicity (Metz *et al.* (2003) *Vaccine* 22, 156-67; Gordon *et al.* (1992) *Infect. Immun.* 60, 485-90) or potential distortion of the native holotoxin structure such that neutralizing antibodies are not generated or are of low titer. Some reports in the literature suggest that cross-
5 neutralization has been achieved in animals vaccinated with chemically prepared Shiga toxoids (Bielaszewska *et al.* (1997) *Infect. Immun.* 65, 2509-16; Ludwig *et al.* (2002) *Can. J. Microbiol.* 48, 99-103); however, the potential for life-threatening toxicity of such a vaccine precludes the use of chemical Stx toxoids in humans. A safer alternative to chemically derived Stx toxoids is the construction of genetic toxoids through the introduction of specific mutations in the Stx A
10 subunit genes to change key amino acids of the enzymatically active domain. Hybrid Stx1 and Stx2 toxins have been made for functional studies of Stxs (Head *et al.* (1991) *J. Biol. Chem.* 266, 3617-3621; Weinstein *et al.* (1989) *Infect. Immun.* 57, 3743-50; Melton-Celsa *et al.* (2002) *Molecular Microbiology* 43, 207-215), including operon fusions allowing A and B subunit expression as a single operon (Weinstein *et al.*, *supra*). Genetic toxoids of Stx1 or Stx2 that
15 protect animals from subsequent lethal challenges of either Stx1 or Stx2 have previously been made (Gordon *et al.* (1992) *Infect. Immun.* 60, 485-90; Ishikawa *et al.* (2003) *Infect. Immun.* 71, 3235-9; Wen *et al.* (2006) *Vaccine* 24, 1142-8). However, such genetic toxoids are unable to circumvent the lack of cross-neutralization between the Stx1 and Stx2 serogroups and only protect against the Stx group from which they were made. To date, there has been no report in
20 the literature of Stx hybrid toxoids being generated.

Summary of the Invention

The invention encompasses a chimeric protein comprising at least one StxA polypeptide or a fragment thereof with one or more modifications at one or more active sites, and at least one
25 StxB polypeptide. In some embodiments, the chimeric protein exists as a pentamer. In further embodiments, the StxB polypeptide or fragment thereof comprises one or more modifications wherein the one or more modifications is an amino acid substitution, addition and/or deletion. In some embodiments, the substitution is a conservative amino acid substitution. In further embodiments, the StxA polypeptide is StxA2 or a fragment thereof and/or the StxB polypeptide
30 is StxB1 or a fragment thereof. In another embodiment, the protein comprises or consists of the amino acid sequence of SEQ ID NO: 2 and/or 3.

In further embodiments, the chimeric proteins of the invention comprise one or more modifications at an amino acid residue in the Stx2A polypeptide or fragment thereof corresponding to residue 77, 167 or 170. In some embodiments, the modification at residue 77 is
35 the substitution of a serine residue while the modification at residue 167 is the substitution of a

044508-5015-WO
2694582.2

glutamine, asparagine or other amino acid residue and the modification at residue 170 is the substitution of a leucine residue. In some embodiments, the modification is capable of reducing or eliminating the enzymatic activity of the Stx2A polypeptide as described herein. In some
5 Stx2A polypeptide as described herein. In other embodiments, the modifications render the protein non-toxic to mammals.

The invention also encompasses an isolated antibody that binds to the chimeric toxoid proteins of the invention and as described herein. In some embodiments, the antibody is a polyclonal antibody. The invention also encompasses a composition comprising one or more of
10 any of the chimeric toxoid proteins of the invention as described herein. In some embodiments, the chimeric protein is capable of inducing an immunogenic response, including, but not limited to, an immunogenic response to Shiga toxin. In some embodiments, the composition further comprises one or more pharmaceutically acceptable carrier(s) and/or adjuvant(s). In another embodiment, the composition is suitable for administration to a human.

15 The invention further encompasses an isolated nucleic acid molecule that encodes the chimeric toxoid proteins of the invention as described herein. In some embodiments, the isolated nucleic acid molecule encodes an amino acid sequence comprising SEQ ID NO: 2 and/or 3. In additional embodiments, the nucleic acid molecule comprises or consists of the nucleotide sequence of SEQ ID NO: 1. In some embodiments, the nucleotide sequence exhibits at least
20 90%, 95% or even up to 99% sequence identity with the contiguous nucleotide sequence of SEQ ID NO: 1 and encodes a polypeptide which is capable of inducing an immunogenic response to Stx.

The invention also encompasses a host cell transformed to contain the nucleic acids of the invention as described herein and includes a vector comprising the isolated nucleic acids.
25 The invention also includes a host cell comprising the aforementioned vector. In some embodiments, the host is selected from the group consisting of prokaryotic and eukaryotic hosts. The invention further encompasses a method of producing a polypeptide comprising culturing a host cell transformed with the nucleic acid molecule of the invention under conditions in which the protein encoded by the nucleic acid molecule is expressed.

30 The invention includes a method of generating antibodies capable of binding to Stx comprising administering a chimeric toxoid protein of the invention as described herein to a mammal or cell culture. The invention further includes a method of generating antibodies capable of binding to Stx comprising administering the composition comprising the chimeric toxoid proteins of the invention to a mammal. In some embodiments, the mammal is a human.
35 In additional embodiments, the human is suffering from diarrhea and/or hemolytic uremic

044508-5015-WO
2694582.2

syndrome.

The invention also encompasses a method of preventing hemolytic uremic syndrome in a human comprising administering a composition comprising the chimeric toxoid proteins of the invention. The invention encompasses a method of preventing diarrhea associated with Shiga toxin-producing *Escherichia coli* infection in a human comprising administering the
5 aforementioned composition. The invention also includes an isolated antibody produced by the any of these methods and a kit comprising this antibody. In some embodiments, the kit further comprises one or more chimeric toxoid proteins of the invention.

10 Brief Description of the Drawings

Figure 1: Western blot analysis of chimeric StxA2/StxB1 toxoid. Wild-type Stx1, Stx2 and chimeric StxA2/StxB1 toxoid (300 ng each) were separated on a 15% SDS-polyacrylamide gel and probed with monoclonal antibody (MAb) hybridoma supernatants directed against StxB1 and StxA2 (13C4 and 11E10 MAbs, respectively). Lane 1 contains Stx1, lane 2 contains Stx2
15 and lane 3 contains the StxA2/StxB1 toxoid.

Figure 2: Detection of anti-Stx1 or anti-Stx2 antibodies by ELISA. Serum IgG titers to Stx1 (left) or Stx2 (right) from mice immunized with either PBS (groups A and C) or the StxA2/StxB1 toxoid (groups B, D and E) are shown in Figure 2. The horizontal bars represent the geometric mean of the log of the IgG serum titer to Stx1 or Stx2 and the error bars indicate \pm
20 one standard deviation. The shaded circles in groups E represent the mouse that died when challenged with Stx1 and Stx2. The dashed line represents the limit of detection.

Figure 3: *In vitro* Stx1 and Stx2 toxin neutralization assays. *In vitro* neutralization titers to Stx1 (left) and Stx2 (right) with antisera from mice immunized with either PBS (groups A and C) or the StxA2/StxB1 toxoid (groups B, D, and E) are shown in Figure 3. The horizontal bars
25 represent the geometric mean of the log of the neutralization titer to Stx1 or Stx2, and the error bars indicate \pm one standard deviation. The amount of Stx1 and Stx2 used was 20 and 38 50% cytotoxic doses (CD_{50}), respectively. The shaded circle in groups E represents the mouse that died when challenged with Stx1 and Stx2. The dashed line represents the limit of detection.

30 Detailed Description

A goal of immunization against Stx and hemolytic uremic syndrome (HUS) is to induce neutralizing antibody responses (NA) broadly reactive against multiple types of Stxs including but not limited to, Stx 1 and 2, and variants of Stx1 and Stx2. The present inventors have studied Stx-producing *E. coli* (STEC) and determined that creation of a chimeric Shiga toxoid with
35 modifications at one or more sites in this hybrid toxoid induces the production of broadly cross-

044508-5015-WO
2694582.2

reactive species of antibodies against Stx following immunization. The invention therefore encompasses chimeric Shiga toxoids, methods of use, and compositions. As used herein, "toxoid" refers to an inactivated holotoxin. As used herein, "enzymatically-inactivated StxA2 subunit" refers to a StxA2 subunit that has lost its functionality through mutations such as *e.g.*, substitutions, additions, and/or deletions.

Shiga Toxoid Chimeric Proteins and Method of Use

The invention encompasses a chimeric Shiga toxoid protein that contains an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit. The StxA2 subunit is inactivated at one or more sites either singly or in combination by site-directed mutagenesis. In some embodiments, the amino acids are deleted while in some embodiments, the amino acids are substituted. Notwithstanding the deletion(s) and/or substitution(s), the conformation of the StxA2 subunit remains sufficiently intact to induce antibodies to multiple subunits of Stxs following administration to a mammal. Mammals, including but not limited to, humans, and/or mice that are immunized with this modified chimeric toxoid develop an immune response, which will reduce or block HUS or other effects of STEC infection or Stx intoxication.

Exemplary suitable substitution sites in the StxA2 protein include, but are not limited to amino acids in StxA2 corresponding to residues 77, 167 and 170 of SEQ ID NO: 2. The substitutions may be Y77S, E167Q and R170L or equivalents. In one embodiment of the invention, the invention encompasses amino acid sequences as set forth in SEQ ID NO: 2 and 3 and fragments thereof. In another embodiment of the invention, the invention encompasses a chimeric Shiga toxoid that contains an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit of the amino acid sequences as set forth in SEQ ID NO: 2 and/or 3 and fragments thereof.

In some embodiments of the invention, the StxB1 subunit does not bind to, or has limited binding to the GB3 receptor but is capable of evoking a protective antibody response.

The invention includes chimeric and/or fusion polypeptides and salts thereof, comprising at least one Shiga toxoid protein and at least one second polypeptide. In some embodiments, the second polypeptide includes a second type of Stx.

The second polypeptide can also include a stabilization domain, which increases the *in vitro* and *in vivo* half-life of the fusion polypeptide. As used herein, the term "stabilization domain" refers to an amino acid sequence capable of extending the *in vitro* and *in vivo* half-life of a Shiga toxoid when compared to the Shiga toxoid alone. The stabilization domain can comprise human proteins (*e.g.*, full length or truncated, soluble proteins from extracellular fragments, etc) such as human serum albumin, transferrin, or other proteins, which stabilize the

044508-5015-WO
2694582.2

in vivo or *in vitro* half-life of the chimeric toxoid protein. These additional functional domains may themselves serve as linker peptides, for example, for joining a Shiga toxoid to a second protein. Alternatively, they may be located elsewhere in the fusion molecule (*e.g.*, at the amino or carboxy terminus thereof). In alternative embodiments, the stabilization domain is a chemical moiety (*e.g.*, PEG (polyethylene glycol) or a dextran).

The term "chimeric" or "fusion polypeptide" as used herein refers to polypeptides in which: (i) a given functional domain (*i.e.* a Shiga toxoid) is bound at its carboxy terminus by a non-covalent bond either to the amino terminus of a second protein (*i.e.*, a second Shiga toxoid) or to a linker peptide which itself is bound by a non-covalent bond to the amino terminus of the second protein; (ii) a given functional domain (*i.e.* a Shiga toxoid) is bound at its amino terminus by a non-covalent bond either to the carboxy terminus of a second protein (*i.e.*, a second Shiga toxoid) or to a linker peptide which itself is bound by a non-covalent bond to the carboxy terminus of the second protein; and/or (iii) the Stxs exist as complex holotoxins with an ABS composition as described herein and having an enzymatically active (A) subunit and a binding domain (B) composed of five identical B proteins that form a pentamer.

Similarly, "fused" when used in connection with the nucleic acid intermediates of the invention means that the 3'- [or 5'-] terminus of a nucleotide sequence encoding a protein is bound to the respective 3'- [or 5'-] terminus of a nucleotide sequence encoding a second protein, either by a covalent bond or indirectly via a nucleotide linker which itself is covalently bound preferably at its termini to the first functional domain-encoding polynucleotide and optionally, a second functional domain-encoding nucleic acid.

Examples of chimeric or fusion polypeptides of the invention may be represented by, but are not limited by, the following formulas:

- R1-L-R2 (i)
- R2-L-R1 (ii)
- R1-L-R2-L-R1 (iii)
- R1-L-R1-L-R2 (iv)
- R2-L-R1-L-R1 (iv)

wherein R1 is the amino acid sequence of a first Shiga toxoid, R2 is the amino acid sequence of a second Shiga toxoid or a stabilizing domain (*e.g.*, human serum albumin), each L is a linker peptide which is bound by a covalent bond to a terminus of R1 and/or R2, whereby the above molecule fragments are read directionally (*i.e.*, with the left side corresponding to the amino terminus and the right side to the carboxy terminus of the molecule). In another embodiment, all or part of an intimin protein (Carvalho *et al.* (2005) Infect. Immun. 73, 2541-2546), that includes the intimin binding domain (carboxy terminus), is R1 and R2 is the chimeric toxoid protein.

044508-5015-WO
2694582.2

Nucleic Acid Molecules and Methods of Use

The present invention further provides nucleic acid molecules that encode the chimeric Shiga toxoid proteins of the invention including a chimeric protein with an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit or fragments thereof, preferably in isolated form. As used herein, "nucleic acid" is defined as RNA or DNA that encodes a protein or peptide as defined above, is complementary to a nucleic acid sequence encoding such peptides, hybridizes to nucleic acid molecules that encode an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit across the open reading frame under appropriate stringency conditions or encodes a polypeptide that shares at least about 65% identity with inactivated StxA2 and about 91% identity with StxB1, alternatively at least about 90% identity with inactivated StxA2 and about 91% identity with StxB1, alternatively at least about 99% identity with inactivated StxA2 and about 95% identity with StxB1, alternatively at least 99.4% identity with inactivated StxA2 and about 99% identity with StxB1.

The nucleic acids of the invention further include nucleic acid molecules that share at least about 90%, alternatively at least about 95%, alternatively at least about 98%, alternatively at least about 99% or more identity with the contiguous nucleotide sequence of nucleic acid molecules that encode the chimeric Shiga toxoid that contains an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit including SEQ ID NO: 2 and/or 3.

In some embodiments of the invention, the nucleic acid molecules contain double or triple base substitutions in the coding region for the StxA2 gene in codons encoding suitable substitution sites such as *e.g.*, in the codons encoding amino acid residues 77, 167 and 170 of the StxA2 protein (SEQ ID NO: 2).

In another embodiment, the nucleic acid molecules contain modifications (*e.g.*, substitutions, additions, and/or deletions) in the coding region for the StxB1 gene that prevent the StxB1 protein from interacting with the kidney cells (via *e.g.*, the host cell GB3 receptor). In one embodiment, such a modification is in one or more codons encoding amino acid residues 16 and/or 17 of the StxB1 protein. These modifications may give rise to amino acid substitutions of D16H and D17H or equivalents. In another embodiment, such a modification is in one or more codons encoding amino acid residues 33, 43, and/or 60 of the StxB1 protein. These modifications may give rise to amino acid substitutions of R33C, A43T, and G60D or equivalents. In yet another embodiment, such a modification is in one or more codons encoding amino acid residues 16 and/or 17 of StxB1 and in one or more codons encoding amino acid residues 33, 43, and/or 60 of the StxB1 protein. These modifications may give rise to amino acid substitutions of D16H and D17H or equivalents and amino acid substitutions of R33C, A43T, and G60D or equivalents.

044508-5015-WO
2694582.2

Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbones, or including alternative bases, whether derived from natural sources or synthesized. Such nucleic acids, however, are defined further as being novel and unobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to nucleic acid encoding a protein according to the present invention. Homology or identity at the nucleotide or amino acid sequence level is determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Altschul *et al.* (1997) *Nucleic Acids Res.* 25, 3389-3402 and Karlin *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 2264-2268, both fully incorporated by reference), which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments, with and without gaps, between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a pre-selected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, please see Altschul *et al.* (1994) *Nature Genetics* 6, 119-129 which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter (low complexity) are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89, 10915-10919, fully incorporated by reference), recommended for query sequences over 85 in length (nucleotide bases).

For blastn, the scoring matrix is set by the ratios of M (*i.e.*, the reward score for a pair of matching residues) to N (*i.e.*, the penalty score for mismatching residues), wherein the default values for M and N are +5 and -4, respectively. Four blastn parameters were adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every winkth position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent Blastp parameter settings were Q=9; R=2; wink=1; and gapw=32. A Bestfit comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

“Stringent conditions” are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C to 68°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50

044508-5015-WO
2694582.2

mM sodium phosphate buffer (pH 6.5) with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is hybridization in 50% formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2× SSC and 0.1% SDS or 68°C in 0.1× SSC and 0.5% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal. Preferred molecules are those that hybridize under the above conditions to the complement of SEQ ID NO: 1 and which encode a functional protein. Even more preferred hybridizing molecules are those that hybridize under the above conditions to the complement strand of the open reading frame of the nucleic acid encoding the chimeric Shiga toxoid that contains an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit. As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules encoding other polypeptides.

The nucleic acid molecule encoding a chimeric Shiga toxoid containing an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit, are part of an operon. One embodiment of the invention is an operon fusion composed of a nucleic acid encoding an enzymatically-inactivated StxA2 subunit followed by a nucleic acid molecule encoding the native *stxB1* intergenic region that contains the ribosomal binding site for translation of StxB1 and then a nucleic acid molecule encoding native StxB1.

The present invention further provides for fragments of the encoding nucleic acid molecule that contain a chimeric Shiga toxoid containing an enzymatically-inactivated StxA2 subunit and the native StxB1 subunit. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the entire protein coding sequence. The size of the fragment will be determined by the intended use. For instance, fragments which encode peptides corresponding to predicted antigenic regions may be prepared. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the encoding nucleic acid molecules of the present invention (*i.e.*, synthetic oligonucleotides) that are used to synthesize gene sequences encoding proteins of the invention, can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci *et al.* (1981) J. Am. Chem. Soc. 103, 3185-3191 or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well-known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene. In one embodiment, the nucleic acid molecule of the present invention contains a contiguous open

044508-5015-WO
2694582.2

reading frame of at least about 1,253 nucleotides, this sequence starting with the optimized Shine-Dalgarno sequence and ending after the *stxB*₁ stop codon.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. The encoding nucleic acid molecules of the present invention may further be modified to contain a label for isolation such as *e.g.*, by adding repeat codons encoding histidine molecules for use in *e.g.*, nickel affinity purification. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides, and the like. A skilled artisan can readily employ any such label to obtain labeled variants of the nucleic acid molecules of the invention. Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein.

In one embodiment, a six histidine tag is added to the C-terminus of the native or slightly modified StxB1 protein to aid in purification of the StxA2/StxB1 toxoid by *e.g.* nickel affinity methods. In another embodiment, a six histidine tag is added to the C-terminus of the native or slightly modified StxB1 protein and six histidine tag is also added to the StxA2 protein. The histidine tag in the StxA2 protein may be located in the immediate vicinity of two histidines present at positions 244 and 245 of StxA2. The histidine tag may be added by making up to four amino acid changes in the StxA2 protein such as *e.g.*, Q246H, G247H, A248H and R249H. Advantageously, the individual tagging of the StxA2 and StxB1 subunits allows for better purification of the subunits using ion exchange and size exclusion purifications procedures in conjunction with the nickel-affinity column purification.

Recombinant Nucleic Acids and Methods of Use

The present invention further provides recombinant nucleic acid molecules (*e.g.*, DNA, RNA) that contain a coding sequence for a chimeric enzymatically-inactivated StxA2 subunit and the native StxB1 subunit. As used herein, a "recombinant nucleic acid molecule" is a nucleic acid molecule that has been subjected to molecular manipulation *in situ*. Methods for generating recombinant nucleic acid molecules are well known in the art, for example, see Sambrook *et al.* (2001) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press. In the preferred recombinant nucleic acid molecules, a coding nucleotide sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the protein family encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell to

044508-5015-WO
2694582.2

be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably, also expression, of the structural gene included in the recombinant nucleic acid molecule.

5 Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

10 In one embodiment, the vector containing a coding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug
15 resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or bacteriophage promoter capable of directing the expression (transcription and translation) of the coding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and
20 transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical examples of such vector plasmids are pBluescript II KS(-) (Stratagene), pTrcHis2C (Invitrogen), pUC8, pUC9, pBR322 and pBR329 (BioRad), pPL and pKK223 (Pharmacia).

25 Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form recombinant nucleic acid molecules that contain a coding sequence. Eukaryotic cell expression vectors, including viral vectors, are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such
30 vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies Inc.), pTDT1 (ATCC), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the recombinant nucleic acid molecules of the present invention may further include a selectable marker that is effective in an
35 eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance

044508-5015-WO
2694582.2

marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene (Southern *et al.* (1982) J. Mol. Anal. Genet. 1, 327-341).

Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug
5 for the selectable marker. The present invention further provides host cells transformed with a nucleic acid molecule that encodes a protein of the present invention. The host cell can be either prokaryotic or eukaryotic.

Eukaryotic cells useful for expression of a chimeric protein of the invention are not limited, so long as the cell line is compatible with cell culture methods and compatible with the
10 propagation of the expression vector and expression of the gene product. Suitable eukaryotic host cells include, but are not limited to, yeast, insect, and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey, or human cell line. Exemplary eukaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells (NIH-3T3) available from the ATCC as CRL 1658, baby hamster
15 kidney cells (BHK), and the like eukaryotic tissue culture cell lines.

Any prokaryotic host can be used to express a recombinant nucleic acid molecule encoding a chimeric protein of the invention. In one embodiment, the prokaryotic host is *E. coli* such as strain DH5 α or BL21. In alternate embodiments, the prokaryotic host is a live attenuated
20 oral bacterial vaccine strain, such as *Shigella flexneri* (Barry *et al.* (2003) Vaccine 21, 333-40) or *V. cholerae* (Leyten *et al.* (2005) Vaccine 23, 5120-5126).

Transformation of appropriate cell hosts with an rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.* (1972) Proc.
25 Natl. Acad. Sci. USA 69, 2110; and Sambrook *et al.* (2001) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press. With regard to transformation of vertebrate cells with vectors containing rDNA, electroporation, cationic lipid or salt treatment methods are typically employed, *see*, for example, Graham *et al.* (1973) Virology 52, 456; Wigler *et al.* (1979) Proc. Natl. Acad. Sci. USA 76, 1373-1376.

30 Successfully transformed cells, *i.e.*, cells that contain a recombinant nucleic acid molecule of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of a recombinant nucleic acid of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their nucleic acid content examined for the
35 presence of the recombinant nucleic acid using a method such as that described by Southern

044508-5015-WO
2694582.2

(1975) J. Mol. Biol. 98, 503-504 or Berent *et al.* (1985) Biotech. 3, 208-209 or the proteins produced from the cell assayed via an immunological method.

Production of Recombinant Proteins

- 5 One skilled in the art would know how to make recombinant nucleic acid molecules which encode chimeric Shiga toxoids of the invention. Furthermore, one skilled in the art would know how to use these recombinant nucleic acid molecules to obtain the proteins encoded thereby, as described herein for the recombinant nucleic acid molecule, which encodes a hybrid Shiga toxoid.
- 10 In accordance with the invention, numerous vector systems for expression of the hybrid Shiga toxoid may be employed. For example, one class of vectors utilizes DNA elements, which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells, which have stably integrated the DNA into their chromosomes,
- 15 may be selected by introducing one or more markers, which allow for the selection of transfected host cells. The marker may provide, for example, prototrophy to an auxotrophic host, biocide resistance, (*e.g.*, antibiotics) or resistance to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by co-transformation. Additional elements may also be needed for
- 20 optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama (1983) Mol. Cell. Biol. 3, 280-289.

- 25 The hybrid Shiga toxoid may be produced by (a) transfecting a cell with an expression vector encoding the hybrid Shiga toxoid; (b) culturing the resulting transfected cell under conditions such that the hybrid Shiga toxoid is produced; and (c) recovering the hybrid Shiga toxoid from the cell culture media or the cells themselves.

- Once the expression vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors may be transfected or introduced into an
- 30 appropriate eukaryotic or prokaryotic cell host. Various techniques may be employed to achieve this, such as, for example, protoplast fusion, calcium phosphate precipitation, electroporation or other conventional techniques. In the case of protoplast fusion, the cells are grown in media and screened for the appropriate activity.

- Methods and conditions for culturing the resulting transfected cells and for recovering
- 35 the hybrid Shiga toxoid so produced are well known to those skilled in the art, and may be varied

044508-5015-WO
2694582.2

or optimized depending upon the specific expression vector and host cell employed.

The host cell for expressing the hybrid Shiga toxoid may be prokaryotic or eukaryotic. Exemplary prokaryotic hosts include *E. coli*, such as *E. coli* DH5 α or BL21. Exemplary eukaryotic hosts include baculovirus vector/insect cell expression systems, yeast shuttle
5 vector/yeast cell expression systems. Methods and conditions for purifying the hybrid Shiga toxoid from the culture media are provided in the invention, but it should be recognized that these procedures can be varied or optimized as is well known to those skilled in the art.

The hybrid Shiga toxoid proteins of the present invention may also be prepared by any known synthetic techniques. Conveniently, the proteins may be prepared using the solid-phase
10 synthetic technique initially described by Merrifield (1965), which is incorporated herein by reference. Other peptide synthesis techniques may be found, for example, in Bodanszky *et al.* (1976), Peptide Synthesis, Wiley.

Immunogenic Compositions and Uses Thereof

15 The chimeric hybrid Shiga toxoid of the invention may be used in a vaccine, immunogenic or pharmaceutical composition to generate an immune response against a Stx. The chimeric Shiga toxoid can also be used in combination with other less immunogenic compositions to assist in eliciting an immune response against the immunogenic compositions. Generally, when used in combination with another immunogenic composition, the immunogenic
20 composition by itself is not sufficient to elicit an immune response and provide a protective effect against a pathogen. In one embodiment, the Shiga toxoids of the invention are used in combination with diphtheria toxin as a means for providing a protective effect against infection by *Corynebacterium diphtheriae*.

In one embodiment, the hybrid Shiga toxoid is used with a suitable adjuvant, as generally
25 understood in the art. Currently, adjuvants approved for human use in the United States include *e.g.*, aluminum salts (alum). These adjuvants have been useful for some vaccines including hepatitis B, diphtheria, polio, rabies, and influenza. Other useful adjuvants include Complete Freund's Adjuvant (CFA), Muramyl dipeptide (MDP), synthetic analogues of MDP, N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanine-2-[1,2-dipalmitoyl-s-glycero-3-(hydroxyph
30 osphoryloxy)]ethylamide (MTP-PE), Incomplete Freund's Adjuvant (IFA), and compositions containing a degradable oil and an emulsifying agent, wherein the oil and emulsifying agent are present in the form of an oil-in-water emulsion having oil droplets substantially all of which are less than one micron in diameter.

The formulation of a vaccine, immunogenic or pharmaceutical compositions of the
35 invention will employ an effective amount of the chimeric Shiga toxoid. That is, there will be

044508-5015-WO
2694582.2

included an amount of antigen which, in combination with the adjuvant, will cause the subject to produce a specific and sufficient immunological response so as to impart protection to the subject from subsequent exposure to Stx. When used as an immunogenic composition, the formulation will contain an amount of antigen, which, in combination with the adjuvant, will cause the subject to produce specific antibodies, which may be used for diagnostic or therapeutic purposes.

5 The vaccine, immunogenic or pharmaceutical compositions of the invention may be useful for the prevention or therapy of hemolytic uremic syndrome (HUS) and/or for the treatment of diarrhea. In one embodiment, the vaccine and/or immunogenic composition are used for the prevention of HUS in the elderly and children. In another embodiment, the vaccine and/or immunogenic composition are used for the prevention of HUS or other consequences of STEC infection or Stx intoxication caused by acts of terrorism, especially for administration to military personnel. Often, more than one administration may be required to bring about the desired prophylactic or therapeutic effect; the exact protocol (dosage and frequency) can be established by standard clinical procedures.

15 The hybrid Shiga toxoid or pharmaceutical compositions comprising the hybrid Shiga toxoid of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal or buccal routes. Alternatively, or concurrently, administration may be by the oral route. In one embodiment particularly suitable for children, a pharmaceutical composition comprising the Shiga toxoid is administered by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

20 The pharmaceutical compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations, which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, for example, sodium carboxymethyl cellulose, sorbitol and dextran. Optionally, the suspension may also contain stabilizers.

30 The precise amounts and formulations for use in either prevention or therapy can vary depending on the circumstances of the inherent purity and activity of the antigen, any additional ingredients, or carriers, the method of administration and the like.

044508-5015-WO
2694582.2

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral, or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

5 Topical administration may be used. Any common topical formulation such as a solution, suspension, gel, ointment or salve and the like may be employed. Preparation of such topical formulations are described in the art of pharmaceutical formulations as exemplified, for example, by Gennaro *et al.* (2000) Remington's Pharmaceutical Sciences, Mack Publishing. For topical application, the compositions could also be administered as a powder or spray,
10 particularly in aerosol form.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

By way of non-limiting illustration, the vaccine dosages administered will typically be,
15 with respect to the antigen, a minimum of about 0.1 mg/dose, more typically a minimum of about 1 mg/dose, and often a minimum of about 10 mg/dose. The maximum dosages are typically not as critical. Usually, however, the dosage will be no more than 500 mg/dose, often no more than 250 mg/dose. These dosages can be suspended in any appropriate pharmaceutical vehicle or carrier in sufficient volume to carry the dosage. Generally, the final volume, including carriers,
20 adjuvants, and the like, typically will be at least 0.1 ml, more typically at least about 0.2 ml. The upper limit is governed by the practicality of the amount to be administered, generally no more than about 0.5 ml to about 1.0 ml.

In an alternative format, vaccine, immunogenic or pharmaceutical compositions may be prepared as vaccine vectors, which express the chimeric hybrid Shiga toxoid or fragment thereof
25 in the host animal. Any available vaccine vector may be used, including live Venezuelan Equine Encephalitis virus (*see* U.S. Patent 5,643,576), poliovirus (*see* U.S. Patent 5,639,649), pox virus (*see* U.S. Patent 5,770,211) and vaccinia virus (*see* U.S. Patents 4,603,112 and 5,762,938). Alternatively, naked nucleic acid encoding the protein or fragment thereof may be administered directly to effect expression of the antigen (*see* U.S. Patent 5,739,118).

30 In one embodiment of the invention, a nucleotide encoding the hybrid Shiga toxoid is transformed into a live attenuated oral bacterial vaccine strain such as *e.g.* *Shigella flexneri* (Barry *et al.* (2003) Vaccine 21, 333-40) or *V. cholerae* (Leyten *et al.* (2005) Vaccine 23, 5120-5126). Thus, such oral bacterial vaccine strains would have expanded protective coverage to include the Stx.

35 In another embodiment of the invention, a nucleotide encoding the hybrid Shiga toxoid is

044508-5015-WO
2694582.2

transformed into a plant to create an edible plant-based vaccine. Alternatively, a nucleotide encoding the hybrid Shiga toxoid could be administered as a DNA-based vaccine. Nucleic acids encoding the chimeric toxoids are administered as DNA vaccines, as either a single genes or combinations of genes. Naked DNA vaccines are generally known in the art (*see* Brower (1998),
5 Nature Biotechnology, 16:1304-1305). Methods for the use of genes as DNA vaccines are well known to one of ordinary skill in the art, and include placing a chimeric toxoid gene or portion thereof under the control of a promoter for expression in a subject in need of treatment. The gene used for DNA vaccines can encode full-length chimeric toxoid proteins, but may also encode portions of the toxoid proteins including peptides derived from the any Shiga toxin gene. The
10 DNA vaccine may contain A and B subunit genes for expression as individual operons under the direction of individual and/or distinct promoters and include rearranging the order of the two coding regions. Modifications made to the nucleotide sequences such as the incorporation of 5' and 3' untranslated regions of viral or eukaryotic origin, polyadenylation signals, codon optimization for optimal expression in a eukaryotic system are also encompassed in the
15 invention. In one embodiment, a subject is immunized with a DNA vaccine comprising a plurality of nucleotide sequences encoding a chimeric toxoid protein. Similarly, it is possible to immunize a subject with a plurality of toxoid genes or portions thereof as defined herein.

In another embodiment, the DNA vaccines include a gene encoding an adjuvant molecule with the DNA vaccine. Such adjuvant molecules include cytokines that increase the
20 immunogenic response to the toxoid protein encoded by the DNA vaccine. Additional or alternative adjuvants are known to those of ordinary skill in the art and find use in the invention. Alternatively, the chimeric toxoid gene itself may serve as an adjuvant in a DNA vaccine containing a nucleic acid encoding another different immunogen.

The hybrid Shiga toxoid may be used in combination, *e.g.*, simultaneously, with vaccines
25 for other diseases. Thus, the hybrid Shiga toxoid may be part of a composition for the treatment and prevention of dysentery and diarrhea, including traveler's diarrhea. Such a composition would be particularly useful for children in third world countries who are exposed to Stx and/or bacteria expressing Stx. The effects of Stx in children tend to be severe leading in some instances to permanent renal damage. Prevention of infection and subsequent exposure to Stx in
30 children is therefore a preferred use for the chimeric Shiga toxoid proteins of the invention.

044508-501S-WO
2694582.2

Antibodies and Methods of Use

This invention further provides for a polyclonal antibody directed to at least one epitope of the StxA2 subunit and at least one epitope of StxB1 subunit and capable of preventing, treating, or diagnosing hemolytic uremic syndrome.

5 The antibodies of the invention may be labeled with a detectable marker. Detectable markers useful in the practice of this invention are well known to those of ordinary skill in the art and may be, but are not limited to radioisotopes, dyes, or enzymes such as peroxidase or alkaline phosphatase, and nanoparticles. Antibodies labeled with detectable markers are particularly useful for diagnosis. The kit may further contain monoclonal or polyclonal anti-StxA2 and anti-
10 StxB1 antibodies that are labeled with a detectable marker and other substituents well known to the art. Such a kit is particularly useful for the detection of Stx or Stx-producing bacteria such as *S. dysenteriae* and *E. coli in vitro* and *in vivo* and for the diagnosis of HUS.

 This invention also concerns an anti-idiotypic antibody directed against the human polyclonal antibodies. This anti-idiotypic antibody may also be labeled with a detectable marker.
15 Suitable detectable markers are well known to those of ordinary skill in the art and may be, but are not limited to radioisotopes, dyes, or enzymes such as peroxidase or alkaline phosphatase.

 The anti-idiotypic antibody is produced when an animal is injected with a polyclonal antibody, which binds to at one epitope of the StxA2 subunit and at least one epitope of StxB1 subunit. The animal will then produce antibodies directed against the idiotypic determinants of
20 the injected antibody (Wasserman *et al.* (1982), Proc. Natl. Acad. Sci. 79, 4810-4814).

 Alternatively, the anti-idiotypic antibody is produced by: (1) contacting lymphoid cells of an animal with an effective-antibody raising amount of the antigen (*i.e.*, the polyclonal antibody which binds to at one epitope of the StxA2 subunit and at least one epitope of StxB1 subunit); (2) collecting the resulting lymphoid cells; fusing the collected lymphoid cells with
25 myeloma cells to produce a series of hybridoma cells, each of which produces a monoclonal antibody; (3) screening the series of hybridoma cells to identify those which secrete a monoclonal antibody capable of binding the resulting hybridoma cell so identified; and (4) separately recovering the anti-idiotypic antibody produced by this cell (Cleveland *et al.* (1983) Nature 305, 56-57). Animals, which may be used for the production of anti-idiotypic antibodies
30 in either of the two above-identified methods, include, but are not limited to humans, primates, mice, rats, or rabbits.

Diagnostic Assays

 The hybrid Shiga toxoid of the present invention may be used as diagnostic reagents in
35 immunoassays to detect anti-Stx antibodies, particularly anti-StxA2 antibodies and anti-StxB1

044508-5015-WO
2694582.2

antibodies. Immunoassay protocols may be based, for example, upon composition, direct reaction, or sandwich-type assays. Protocols may also, for example, be heterogeneous and use solid supports, or may be homogeneous and involve immune reactions in solution. Most assays involved the use of labeled antibody or polypeptide. The labels may be, for example,
5 fluorescent, chemiluminescent, radioactive, nanoparticles, or dye molecules. Assays which amplify the signals from the probe are also known, examples of such assays are those which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Typically, an immunoassay for anti-Stx antibodies will involve selecting and preparing
10 the test sample, such as a biological sample, and then incubating it with a modified hybrid Shiga toxoid of the present invention under conditions that allow antigen-antibody complexes to form. Such conditions are well known in the art. In a heterogeneous format, the protein or peptide is bound to a solid support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose, in membrane or
15 microtiter well form, polyvinylchloride, in sheets or microtiter wells, polystyrene latex, in beads or microtiter plates, polyvinylidene fluoride, diazotized paper, nylon membranes, activated beads, and Protein A beads. Most preferably, Dynatech, Immulon® microtiter plates, or 0.25 inch polystyrene beads are used in the heterogeneous format. The solid support is typically washed after separating it from the test sample.

20 In homogeneous format, on the other hand, the test sample is incubated with the hybrid Shiga toxoid in solution, under conditions that will precipitate any antigen-antibody complexes that are formed, as is known in the art. The precipitated complexes are then separated from the test sample, for example, by centrifugation. The complexes formed comprising anti-Stx antibodies are then detected by any number of techniques. Depending on the format, the
25 complexes can be detected with labeled anti-xenogenic immunoglobulin or, if a competitive format is used, by measuring the amount of bound, labeled competing antibody. These and other formats are well known in the art.

Diagnostic probes useful in such assays of the invention include antibodies to Stx. The antibodies may be either monoclonal or polyclonal produced using standard techniques well
30 known in the art (*see* Harlow & Lane (1988), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press). They can be used to detect Stx by specifically binding to the protein and subsequent detection of the antibody-protein complex by ELISA, Western blot or the like. The hybrid Shiga toxoid used to elicit these antibodies can be any of the variants discussed above. Antibodies are also produced from peptide sequences of the hybrid Shiga toxoid using
35 standard techniques in the art (Harlow & Lane, *supra*). Fragments of the monoclonals or the

044508-5015-WO
2694582.2

polyclonal antisera, which contain the immunologically significant portion, can also be prepared.

The following working examples specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art.

5

Examples

Example 1: Construction of chimeric *stxA₂/stxB₁* and toxoid mutations

A genetically toxoided version of Stx that could be used as a vaccine to protect against both Stx1 and Stx2 was constructed. Because the StxB1 protein is highly immunogenic and
10 more protective than the StxB2 subunit protein (Marcato *et al.* (2001) J. Infect. Dis. 183, 435-43), StxB1 was used for the B subunit portion of the vaccine. StxA2 was chosen for the A subunit of the vaccine construct.

The *stxA₂* and *stxB₁* genes were spliced together to generate an operon fusion composed of *stxA₂* followed by the native *stxB₁* intergenic region that contains the ribosomal binding site for
15 translation of StxB1, then the *stxB₁* gene. The holotoxoid expression cassette was designed in the native operon configuration to optimize translation and assembly of the AB5 holotoxoid.

Next, the StxA2 portion of the chimeric toxoid was modified by introducing three modifications (Y77S, E167Q, and R170L) to prevent a potential for reversion to toxicity and to maximize immunogenicity. The selection of these mutations was based on prior studies that
20 showed that these mutations either reduced the cytotoxicity of the toxin or increased the immunogenicity of a toxoid. The substitution of tyrosine at position 77 to serine was generated because this change substantially reduces the activity of Stx1, and we predicted that the same would be true for Stx2 (Deresiewicz *et al.* (1992) Biochemistry 31, 3272-80; Deresiewicz *et al.* (1993) Mol. Gen. Gen. 241, 467-73). The decision to change the glutamic acid at position 167
25 of StxA2 to glutamine was done because this amino acid is in the active site of both Stx1 and Stx2 and such an alteration leads to a dramatic reduction in the Vero cell activity of the toxin (Gordon *et al.* (1992) Infect. Immun. 65, 2509-16; Hovde *et al.* (1988) Proc. Natl. Acad. Sci. USA 85 2568-72; Jackson *et al.* (1990) J Bacteriol 182, 3346-50; Yamaski *et al.* (1991) Microb. Pathog. 11, 1-9). The choice to replace arginine at position 170 with leucine reflects the
30 Ishikawa *et al.* observation that the StxA1 protein is more immunogenic after such a substitution (Ishikawa *et al.* (2003) Infect Immun 71, 3235-9). A detailed description of the methodology follows.

Bacterial plasmids used are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or on LB agar (Becton Dickinson) supplemented with 100 µg/ml of ampicillin as needed
35 for selection of recombinant plasmids.

044508-S015-WO
2694582.2

Table 1: Cloning and Expression Plasmids	
pBluescript SK II	<i>E. coli</i> cloning vector (Amp ^r)
pTrcHis2 C	<i>E. coli</i> expression vector (Amp ^r)
pCKS112	<i>stx</i> ₁ clone
pJES120	<i>stx</i> ₂ clone
pMJS1	pBluescript II KS (-) clone of <i>stx</i> ₁ from pCKS112
pMJS2	pBluescript II KS (-) clone of <i>stx</i> ₂ from pJES120
pMJS21	pBluescript II KS (-) clone of <i>stxA</i> ₂ / <i>stxB</i> ₁
pMJS22	pMJS21 with StxA2 Y77S mutation
pMJS23	pMJS22 with StxA2 E167Q and R170L mutations
pMJS24	<i>stxA</i> ₂ / <i>stxB</i> ₁ toxoid clone from pMJS23 cloned into pTrcHis2 C
pMJS25	TA-cloning vector pCR2.1 clone of pMJS23 with StxA2 E167Q change from CAA (Q) to CAG (Q)
pMJS26	pTrcHis2 C clone of the <i>stxA</i> ₂ / <i>stxB</i> ₁ toxoid clone made on pMJS25 with six histidines added to the C-terminus of StxB1.
pMJS27	pTrcHis2 C clone of the <i>stxA</i> ₂ / <i>stxB</i> ₁ toxoid clone made on pMJS26 with StxB1 D16H and D17H mutations

- The chimeric *stxA*₂/*stxB*₁ operon was created by a series of polymerase chain reactions (PCR) followed by a splicing by overlap extension (SOE) step (Higuchi *et al.* (1989), PCR Technology, Stockton Press). Specifically, PCR amplification of sequences from pMJS2 with primers MJS5 and MJS32 (see Table 2) was used to synthesize *stxA*₂; similarly, PCR amplification of sequences from pMJS1 with primers MJS20 and MJS2 was used to generate *stxB*₁. After the *stxA*₂ and *stxB*₁ PCR products were spliced together, the chimeric *stxA*₂/*stxB*₁ operon was cloned into pBluescript II KS⁻ (Stratagene) under direction of the *stx*₂ promoter. The resultant plasmid was named pMJS21 and transformed into *E. coli* DH5α.

Table 2: Synthetic oligonucleotide primers (5' to 3')		
Primer	Primer Sequence (5' to 3')	Purpose/Region of homology
MJS1	gatcggatcccccgtgaacgaagttgcgtaacagc (SEQ ID NO: 4)	<i>stx</i> ₁ upstream primer
MJS2	gatcgaattctcgcttacgatcatcaagagatcatacc (SEQ ID NO: 5)	<i>stx</i> ₁ downstream primer
MJS5	gatcggatccagcaagggccaccatcacataccgcc	<i>stx</i> ₂ upstream primer

044508-501S-WO
2694582.2

Table 2: Synthetic oligonucleotide primers (5' to 3')		
Primer	Primer Sequence (5' to 3')	Purpose/Region of homology
	(SEQ ID NO: 6)	
MJS6	caggggaattcacatgcgaaatttttaacaaatgc (SEQ ID NO: 7)	<i>stx₂</i> downstream primer
MJS20	gggggtaaaatgaaaaaacattattaatgc (SEQ ID NO: 8)	Used with MJS32 to generate pMJS21
MJS32	gctattaataatgttttttcattttaccccttattaccggtgtatataaaaactg (SEQ ID NO: 9)	Used with MJS20 to generate pMJS21
MJS88	tcagtggccgggttcgtaataacgg (SEQ ID NO: 10)	Used with MJS89 to generate pMJS22
MJS89	ccgtattaacgaaccggccactgataaattatttgctcaataatcagacgaagatggc (SEQ ID NO: 11)	Used with MJS88 to generate pMJS22
MJS90	caagccttattattcaggcagatacagagagaatttcgtcaggc (SEQ ID NO: 12)	Used with MJS91 to generate pMJS23
MJS91	ctctgtatctgcctgaataataaggcttgctgtgacagtgacaaaacgcagaactgctctgg (SEQ ID NO: 13)	Used with MJS90 to generate pMJS23
2A5SD	gatcgatcctaaggaggacagctatgaagtgtatattttaaatgggtactg (SEQ ID NO: 14)	Used to generate pMJS24, pMJS26, and pMJS27
MJS97	gatcatcgatagccaaaaggaaacacctgtatatg (SEQ ID NO: 15)	<i>stxA₂</i> upstream primer, used to generate pMJS25
MJS98F	gatcgctagctcaacgaaaaataacttcgctgaatcc (SEQ ID NO: 16)	<i>stxB₁</i> downstream primer used to generate pMJS25
MJS92	caggccttattattcaggcag (SEQ ID NO: 17)	Used with MJS93 to generate pMJS25
MJS93	ctgcctgaataataaggcctgtgctgtgacagtgacaaaacgcagaactgctctggatgc (SEQ ID NO: 18)	Used with MJS92 to generate pMJS25
1BC1	ggtggtggtgacgaaaaataacttcgctgaatcc (SEQ ID NO: 19)	<i>stxB₁</i> His-tagged downstream primer #1, used to generate pMJS26 and pMJS27
1BC2	cagtgggtggtggtggtgacgaaaaataac (SEQ ID NO: 20)	<i>stxB₁</i> His-tagged downstream primer #2, used to generate pMJS26 and pMJS27

044508-5015-WO
2694582.2

Table 2: Synthetic oligonucleotide primers (5' to 3')		
Primer	Primer Sequence (5' to 3')	Purpose/Region of homology
1BC3	<u>gatcgaattctcagtggtggtggtggtg</u> (SEQ ID NO: 21)	<i>stxB₁</i> His-tagged downstream primer #3, used to generate pMJS26 and pMJS27
1B2HF	<u>catcacgataccttacagttaagtggg</u> (SEQ ID NO: 22)	Used with 1B2HR to generate pMJS27
1B2HR	<u>ttaactgtaaaggtatcgtgatgattatattgtatactccacc</u> (SEQ ID NO: 23)	Used with 1B2HF to generate pMJS27
Restriction enzyme sites are underlined, Mutagenic codon sites are in bold.		

Next, a set of nucleotide changes were engineered into the *stxA₂* gene of *stxA₂/stxB₁* to generate an operon from which a hybrid toxoid molecule could be expressed. Specifically, the tyrosine at position 77 of the StxA2 mature protein was changed to a serine residue by amplification of the DNA with mutagenic primers MJS88 and MJS89 and flanking primers MJS2 and MJS5 to yield pMJS22. Then the glutamic acid at the active site of StxA2 (residue 167) was altered to a glutamine and the arginine at position 170 was changed to a lysine simultaneously by PCR with mutagenic primers MJS90 and MJS91 and flanking primers MJS2 and MJS5 to yield pMJS23. The chimeric toxoid operon was then amplified from pMJS23 by PCR with the 2A5SD and MJS2 primers to introduce an optimized Shine-Dalgarno sequence (TAAGGAGGACAGCTATG) (SEQ ID NO: 24) upstream of the start codon for StxA2 and to remove the native *stx₂* promoter. The resulting chimeric clone was ligated into the expression vector pTrcHis2 C (Invitrogen) and transformed into *E. coli* strain BL21 (DE3). DNA sequence analysis was done by the Biomedical Instrumentation Center at the Uniformed Services University to verify that the correct mutations were made.

Example 2: Purification of the StxA2/StxB1 toxoid

The purification of the StxA2/StxB1 toxoid was done by Gb3 affinity purification as described previously (Ishikawa *et al.* (2003) Infect. Immun. 71, 3235-9; Wen *et al. supra*). Gb3 affinity purification is a process that captures the B subunit binding domain. An overnight culture of *E. coli* BL21 (DE3) that contained the pTrcHis2 C- *stxA₂/stxB₁* clone was diluted 1:50 into two flasks each containing 500 ml LB broth. After 2 hours of growth, the cultures were induced with 1 mM isopropyl 3-D-thiogalactopyranoside (IPTG) and incubated for an additional 4 hours.

The bacteria were pelleted by centrifugation and concentrated 50-fold by re-suspension

044508-5015-WO
2694582.2

in 10 ml of 1× phosphate-buffered saline, pH 7.4 (PBS). The concentrated bacterial suspensions were then disrupted by sonication and the resulting lysates clarified by centrifugation. The StxA2/StxB 1 toxoid was purified from these lysates by Gb3 affinity purification on Globotriose Fractogel columns (IsoSep AB) as described previously (Ishikawa *et al.*, *supra*; Wen *et al.*,
5 *supra*). After purification of the toxoid, a BCA assay (Pierce) was done to quantify the protein concentration of the preparation.

Example 3: Cytotoxicity Assay

The cytotoxic activity for Vero cells of various samples was assessed as previously
10 described. Briefly, *E. coli* DH5α transformed with toxin clones in pBluescript II KS (-) were normalized to the same optical density (O.D.)₆₀₀, and the bacteria were disrupted by sonication and clarified by centrifugation. Clarified sonic lysates or purified toxoid were assessed for Vero cell cytotoxic activity (Schmitt *et al.* (1991) Infect. Immun. 59, 1065-73; Gentry *et al.* (1980) 26, 2127-31) after diluting the samples in Eagle's minimum essential medium (Cambrex BioScience)
15 supplemented with 9% fetal bovine serum (BioSource International), 1.8 mM glutamine (Cambrex), 9 U/ml of penicillin 9 pg/ml of streptomycin (Invitrogen Corporation), and 90 µg/ml of gentamicin (Quality Biological) (complete EMEM). The 50% cytotoxic dose (CD₅₀) of samples was defined as the reciprocal of the dilution that killed 50% of the Vero cells, as compared to the untreated control cells.

20 The cytotoxic activities of the different versions of the mutated StxA2/StxB1 toxoids for Vero cells were compared to the parent StxA2/StxB1 toxin (see Table 3). While one ml of a clarified sonic lysate of the parent hybrid toxin contained 7.2×10^4 CD₅₀s, similarly prepared lysates of the altered chimeric molecule with either a single mutation (Y77S) or the triple mutations (Y77S, E167Q and R170L) had no demonstrable cytotoxic activity, a finding that
25 confirmed that these constructs produced toxoids. Additionally, 2.1 µg of the purified StxA2/StxB1 toxoid was not cytotoxic to Vero cells; this was approximately 97 times the concentration of the parent StxA2/StxB1 toxin assayed. The quantity of the parent StxA2/StxB1 toxin was determined by Western blot analysis using polyclonal antibody against Stx2 and quantifying StxA2 subunit by comparison with an Stx2 standard. To determine if there was any
30 residual activity at very low levels of the StxA2/StxB1 toxoid, a 100-fold preparation of crude bacterial lysate was tested for cytotoxicity on Vero cells and for toxicity in mice. Five CD-1 male mice were injected intraperitoneally with 86 µg of the toxoid (this corresponds to 20 times the amount used to immunize and boost the mice) and all of the mice survived. Additionally, 61.3 µg of the toxoid from the 100-fold preparation crude bacterial lysate was tested for
35 cytotoxicity and was found to be non-cytotoxic to Vero cells.

044508-5015-WO
2694582.2

Table 3: Results of the Cytotoxic assay	
Toxin or toxoid	Cytotoxicity (Vero cell CD ₅₀ /ml of culture)
pBluescript II (KS-)	$< 1 \times 10^2$
Stx1	8.1×10^6
Stx2	5.5×10^4
StxA2/StxB1	7.2×10^4
StxA2/StxB1 (Y77S)	$< 1 \times 10^2$
StxA2/StxB1 (Y77S, E167Q, R170L)	$< 1 \times 10^2$
StxA2/StxB1 toxoid (purified, 2.1 μ g)	$< 1 \times 10^2$

Toxin lysates were from *E. coli* DH5 α transformed with pMJS1 (Stx1), pMJS2 (Stx2), pMJS21 (StxA2/StxB1), pMJS22 (StxA2/StxB1 with StxA2 Y77S), pMJS23 (StxA2/StxB1 with StxA2 Y77S, E167Q and R170L). The purified StxA2/StxB1 toxoid was purified from *E. coli* BI21. The values represent the reciprocal of the dilution that killed 50% of the Vero cells. The
5 limit of detection for this experiment was 1×10^2 .

Example 4: Western blot analysis

Purified Stx1, Stx2 or the StxA2/StxB1 toxoid (300 ng each) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide) and then the proteins were transferred to 0.45 μ M Optitran nitrocellulose membranes (Schleicher &
10 Schuell) with a Trans-Blot SD Semi-Dry transfer apparatus (Bio-Rad) (see Figure 1). The membranes were blocked overnight at 4°C in a solution of 5% non-fat dry milk in 1 \times Tris-buffered saline (pH 7.5) with 0.1% Tween 20 added (TBST). The primary antibodies, hybridoma tissue culture supernatants diluted 1:5 each in blocking solution of anti-StxA2 and anti-StxB1 monoclonal antibodies (MAbs) 11E10 and 13C4, respectively (Gentry *et al.* (1980) J.
15 Clin. Microbiol. 12, 361-6; Perera *et al.* (1980) J. Clin. Microbiol. 26, 2127-31), were incubated with the immunoblots for two hours. The membranes were then washed in TBST and incubated for one hour with goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (Bio-Rad) at a dilution of 1:3,000 in blocking solution. The membranes were washed again as described above, and the secondary antibody was detected by chemiluminescence with
20 the ECL-Plus Western Blotting Detection kit (Amersham).

The immunoblot of the purified toxoid revealed both StxB1 and StxA2 were present (see Figure 1), a finding that confirmed our supposition that a holotoxoid was expressed by the vaccine construct. In addition, the immunoreactive bands of the toxoid corresponded in size to the native toxin subunit controls (see Figure 1). The StxA2 and StxB1 subunits were also visible

044508-5015-WO
2694582.2

on the silver stained-gel with little evidence of additional contaminating proteins (data not shown).

Example 5: Mouse Immunization and Challenge

5 Pre-immune sera were collected from male CD-1 mice that weighed 14 to 16 grams (Charles River). The mice were then immunized intraperitoneally (i.p.) with either PBS or 4.3 µg of purified StxA2/StxB1 toxoid in PBS mixed 1:1 with TiterMax Gold, a water-in-oil adjuvant (TiterMax USA) (total volume 100 µl). The mice were boosted at three-week intervals, for a total of three boosts. Serum was collected ten days after the initial immunization and after
10 each boost to determine serum immunoglobulin G (IgG) levels against Stx1 or Stx2. The mice were challenged i.p. 14 days after the third boost with 10 times the 50% lethal dose (LD₅₀) of either Stx1 (1,250 ng) or Stx2 (10 ng) or both Stx1 and Stx2 (1,250 and 10 ng/mouse, respectively).

All of the PBS-immunized mice died by day 4, regardless of the Stx type administered
15 (see Table 4). All of the chimeric toxoid-immunized mice subsequently challenged with either Stx1 or Stx2 survived the entire 14 day observation period. Nine of the ten chimeric toxoid-immunized mice that were subsequently challenged with both Stx1 and Stx2 survived. The chimeric toxoid-immunized mouse that succumbed after challenge with lethal doses of both Stx1 and Stx2 was the mouse that also failed to produce anti-Stx1 and anti-Stx2 neutralizing
20 antibodies; this mouse died at approximately the same time as did the mock-immunized animals. The LD₅₀ was previously determined to be 125 and 1 ng/mouse for Stx1 and Stx2. The average weight of the mice when they were challenged was 40.4 g.

Table 4: Protection of immunized mice against a lethal challenge			
Group	immunogen	challenge with 10 LD ₅₀	# survivors per total
A	PBS	Stx1	0/10
B	StxA2/StxB1 toxoid	Stx1	10/10
C	PBS	Stx2	0/10
D	StxA2/StxB1 toxoid	Stx2	10/10
E	StxA2/StxB1 toxoid	Stx1 and Stx2	9/10

Example 6: Determination of anti-Stx1 or anti-Stx2 antibodies by ELISA

25 Male CD-1 mice were immunized and boosted at three-week intervals with either PBS or the triple-mutant toxoid. After the third and final boost, serum from each mouse was collected, and the titers of IgG antibodies against Stx1 and Stx2 were compared to the appropriate pre-

044508-5015-WO
2694582.2

immune serum sample by ELISA (see Figure 2).

Purified Stx1 or Stx2 (100 ng in 100 ml PBS) was used to coat the wells of a U-bottom 96-well microtiter plate (Thermo Electron), and the microtiter plates were incubated at 4°C overnight. The microtiter plates were then washed three times in PBS that contained 0.05% Tween-20 (PBST) and blocked overnight at 4°C with 200 µl per well of PBST that contained 3% bovine serum albumin. The next day, in a separate microtiter plate, the mouse pre- and post-immunization sera were diluted in PBST, with an initial dilution of 1:50 and 1:5 dilutions thereafter. After the blocked microtiter plates were washed, 100 µl of the diluted serum was used as the primary antibody for the ELISAs, and the microtiter plates were incubated for two hours at 37°C. Next, 100 µl of the secondary antibody, goat anti-mouse IgG conjugated to HRP was added at a dilution of 1:3,000 in PBST, and the plates were incubated at room temperature for 45 min. The secondary antibody was detected with the TMB Peroxidase EIA substrate kit (Bio-Rad), and the microtiter plates were incubated at room temperature for 15 minutes to allow for a color change to develop. One hundred µl of 1M H₂SO₄ was then added to quench the reaction, and the color development was determined by measurement of the OD₄₅₀. The ELISA titer was defined as the serum dilution that was above both background and pre-immune levels. In cases where the pre-immune levels were higher than the post-immune levels, a value of 0.3 was assigned as the ELISA titer. These assays were done once in duplicate. The positive controls for the anti-Stx1 and anti-Stx2 ELISAs were either purified 11E10 or 13C4 (Hycult Biotechnology) as the primary antibodies, each diluted 1:20,000 in PBST.

Example 7: In vitro Stx1 and Stx2 toxin neutralization assays

Because toxin neutralization titers are more predictive of a protective response to Stxs than ELISA titers (Wen *et al.*, *supra*), *in vitro* Vero cell neutralization assays against purified Stx1 or Stx2 were also performed on serum samples.

The pre- and post-immunization sera were used in a neutralization assay for Stx1 and Stx2 as reported previously (Marques *et al.* (1986) J. Infect. Dis. 154, 338-41). The neutralization titer was defined as the dilution of the mouse serum that neutralized 50% of the cytotoxicity of Stx1 or Stx2. The amount of Stx1 or Stx2 used in these assays was 20 or 38 CD₅₀s respectively. In cases where the mock-immunized mouse serum or post-immunization serum did not neutralize either Stx1 or Stx2, a value of 0.3 was assigned to the neutralization titer. These assays were done once in duplicate.

Example 8: Comparison of neutralization antibody titers via Statistical Analysis

The anti-Stx1 or anti-Stx2 ELISA and *in vitro* neutralization antibody titers from the

044508-5015-WO
2694582.2

toxoid-immunized groups were compared to the mock-immunized groups by the two-sided t test using the program SPSS 12.0.1. The survival of immunized mice was compared to the mock-immunized mice after challenging with 10 LD₅₀s of either Stx1 or Stx2 or Stx1 and Stx2 by the Fisher's exact test. These results were considered significantly different if the p value was < 0.05.

5 The pre-immune and mock-immunized mice had low background levels of antibodies that reacted with Stx1 or Stx2 (*see* Figure 2). All but one mouse immunized with the toxoid displayed high IgG titers to both Stx1 and Stx2, 4.4 and 4.1 logs above background, respectively. When the ELISA titers of the toxoid-immunized mice were compared to the mock-immunized
10 mice, the results were found to be significant ($p < 0.001$).

No measurable neutralizing antibodies to either Stx1 or Stx2 were detected in pre-immune or mock-immunized mice (*see* Figure 3). In contrast, all but one mouse immunized with the toxoid displayed neutralizing titers to Stx1 and Stx2 (*see* Figure 3). The mean anti-Stx1 and anti-Stx2 neutralization titers were 2.9 and 1.9 logs above background. The lower Stx2-
15 neutralizing titers may be attributable to the higher concentration of Stx2 than Stx1 used in the neutralization assays (about 38 CD₅₀s compared to 20 CD₅₀s, respectively). The toxoid-immunized mouse that showed a poor anti-Stx1 ELISA titer also failed to produce neutralizing antibodies against either toxin. When the neutralization titers of the toxoid-immunized mice were compared to the mock-immunized mice, the results were found to be significantly different
20 ($p < 0.001$).

Although the present invention has been described in detail, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.

044508-5015-WO
2694582.2

What is claimed:

1. A chimeric protein comprising at least one StxA polypeptide or a fragment thereof with one or more modifications at one or more active sites, and at least one StxB polypeptide.
2. The chimeric protein of claim 1, wherein the StxB polypeptide or fragment thereof comprises one or more modifications.
3. The chimeric protein of claim 1 or 2, wherein the one or more modifications is an amino acid substitution or deletion.
4. The chimeric protein of claim 3, wherein the substitution is a conservative substitution.
5. The chimeric protein of claim 1, wherein the StxA polypeptide is StxA2 or a fragment thereof.
6. The chimeric protein of claim 1, wherein the StxB polypeptide is StxB1 or a fragment thereof.
7. The chimeric protein of claim 1, wherein the protein comprises the amino acid sequence of SEQ ID NO: 2 and 3.
8. The chimeric protein of claim 1, wherein the protein consists of the amino acid of SEQ ID NO: 2 and 3.
9. The chimeric protein of claim 5, wherein the one or more modification is at an amino acid residue in the Stx2A polypeptide or fragment thereof corresponding to residue 77, 167 or 170.
10. The chimeric protein of claim 9, wherein the modification at residue 77 is the substitution of a serine residue.
11. The chimeric protein of claim 9, wherein the modification at residue 167 is the substitution of a glutamine residue.
12. The chimeric protein of claim 9, wherein the modification at residue 170 is the substitution of a leucine residue.
13. The chimeric protein of claim 6, wherein the one or more modification is at an amino acid residue in the StxB1 polypeptide corresponding to residue 16, 17, 33, 43 or 60.

044508-5015-WO
2694582.2

14. The chimeric protein of claim 13, wherein the modification at residue 16 is the substitution of a histidine for an aspartate residue.
15. The chimeric protein of claim 13, wherein the modification at residue 17 is the substitution of a histidine for an aspartate residue.
- 5 16. The chimeric protein of claim 13, wherein the modification at residue 33 is the substitution of a cysteine for an arginine residue.
17. The chimeric protein of claim 13, wherein the modification at residue 43 is the substitution of a threonine for an alanine residue.
18. The chimeric protein of claim 13, wherein the modification at residue 60 is the substitution of an aspartate for a glycine residue.
- 10 19. An isolated antibody that binds to the chimeric protein of claim 1.
20. The isolated antibody of claim 19, wherein the antibody is polyclonal.
21. A composition comprising the chimeric protein of claim 1.
22. The composition of claim 21, wherein the chimeric protein is capable of inducing an immunogenic response.
- 15 23. The composition of claim 21, wherein the chimeric protein is capable of inducing an immunogenic response to Shiga toxin.
24. The composition of claim 21 further comprising a pharmaceutically acceptable carrier.
25. The composition of claim 21 further comprising an adjuvant.
- 20 26. The composition of claim 21, wherein composition is suitable for administration to a human.
27. An isolated nucleic acid molecule that encodes the chimeric protein of claim 1.
28. An isolated nucleic acid molecule that encodes an amino acid sequence comprising SEQ ID NO: 2 and/or 3.
- 25 29. The isolated nucleic acid molecule of claim 28, wherein the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 1.

044508-5015-WO
2694582.2

30. The isolated nucleic acid molecule of claim 28, wherein the nucleic acid molecule consists of the nucleotide sequence of SEQ ID NO: 1.
31. A host cell transformed to contain the nucleic acid molecule of claim 27.
32. A vector comprising the isolated nucleic acid molecule of claim 27.
- 5 33. A host cell comprising the vector of claim 32.
34. The host cell of claim 33, wherein said host is selected from the group consisting of prokaryotic host and eukaryotic hosts.
35. A method of producing a polypeptide comprising culturing a host cell transformed with the nucleic acid molecule of claim 33 under conditions in which the protein encoded by the
10 nucleic acid molecule is expressed.
36. The isolated nucleic acid molecule of claim 27 wherein the nucleotide sequence exhibits at least 90% sequence identity with the contiguous nucleotide sequence of SEQ ID NO: 1 and encodes a polypeptide which is capable of inducing an immunogenic response to Stx.
37. The isolated nucleic acid molecule of claim 27 wherein the nucleotide sequence exhibits
15 at least 95% sequence identity with the contiguous nucleotide sequence of SEQ ID NO: 1 and encodes a protein which is capable of inducing an immunogenic response to Stx.
38. The isolated nucleic acid molecule of claim 27 wherein the nucleotide sequence exhibits at least 99% sequence identity with the contiguous nucleotide sequence of SEQ ID NO: 1 and encodes a protein which is capable of inducing an immunogenic response to Stx.
- 20 39. A method of generating antibodies capable of binding to Stx comprising administering a chimeric protein of claim 1 to a mammal.
40. A method of generating antibodies capable of binding to Stx comprising administering a chimeric protein of claim 1 to a cell culture.
41. A method of generating antibodies capable of binding to Stx comprising administering
25 the composition of claim 21 to a mammal.
42. The method of claim 39 or 41, wherein the mammal is a human.
43. The method of claim 42, wherein the human is suffering from diarrhea.

044508-5015-WO
2694582.2

44. The method of claim 42, wherein the human is suffering from hemolytic uremic syndrome.

45. A method of preventing hemolytic uremic syndrome in a human comprising administering a composition comprising the chimeric protein of claim 1.

5 46. A method of preventing diarrhea associated with Shiga toxin-producing *Escherichia coli* infection in a human comprising administering a composition comprising the chimeric protein of claim 1.

47. An isolated antibody produced by the method of claims 39, 40 or 41.

48. A kit comprising an antibody of claim 47.

10 49. A kit comprising the chimeric protein of claim 1.

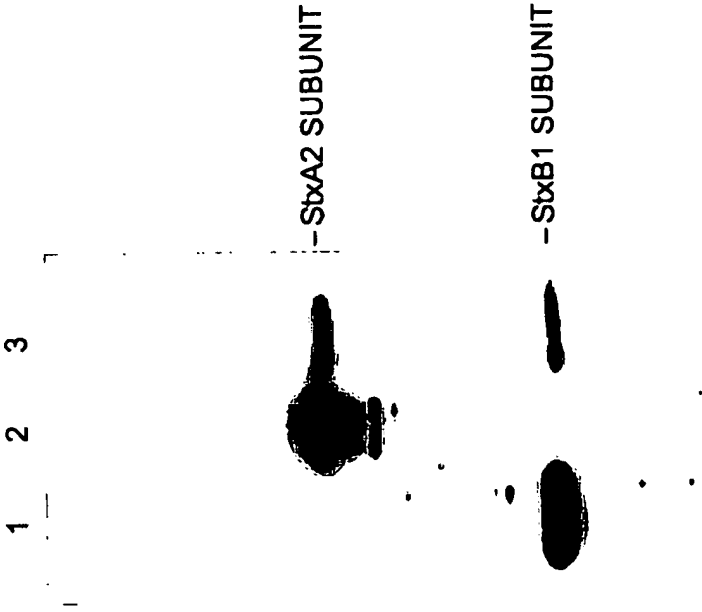


FIG. 1

2/3

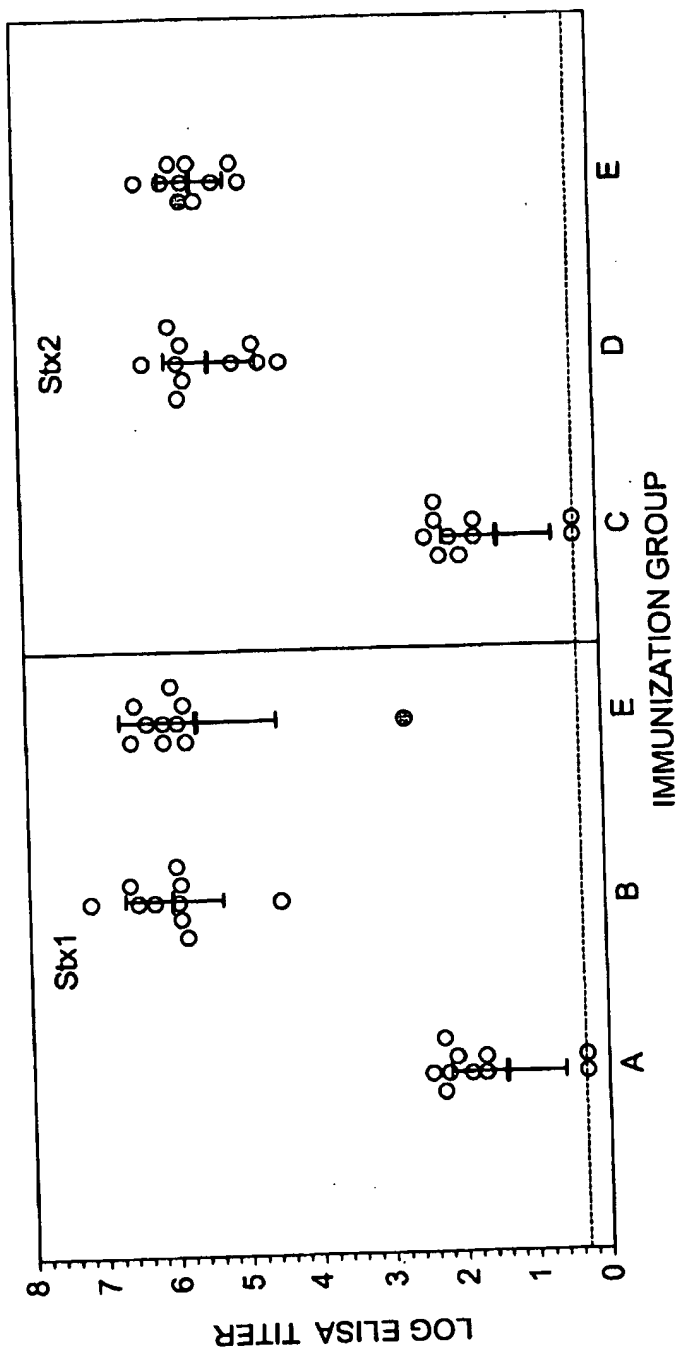


FIG. 2

3/3

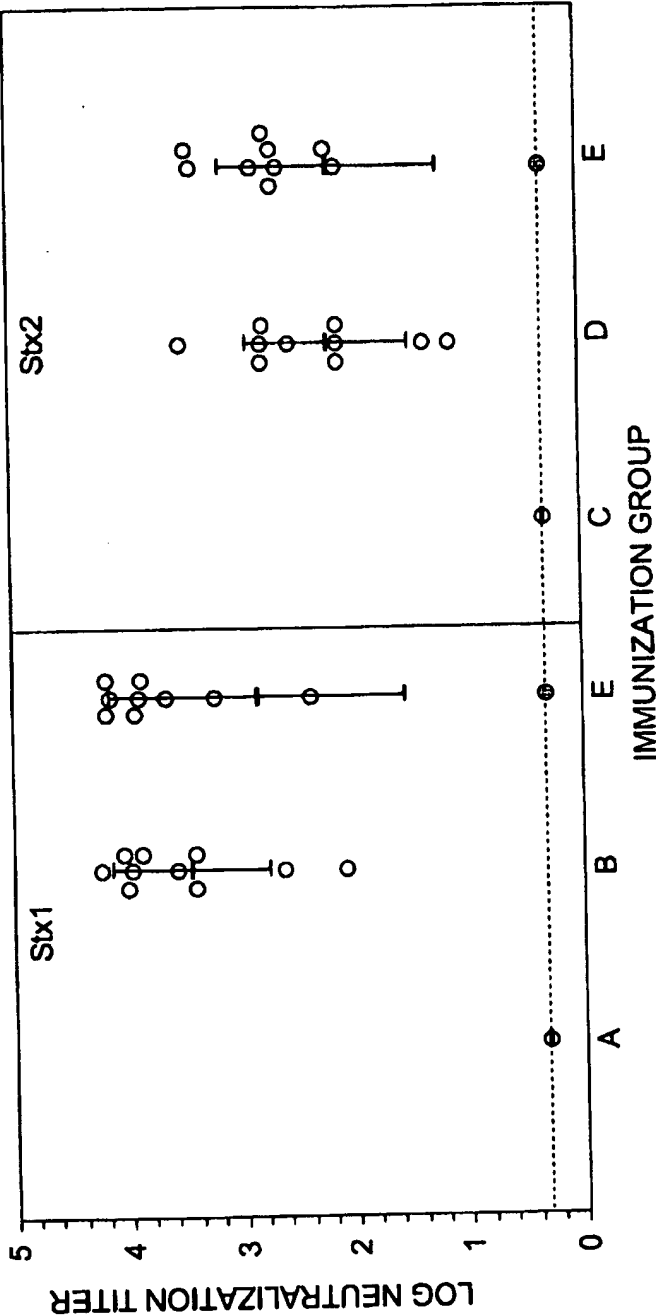


FIG. 3